

DNA Methylation Down-Regulates *EGFR* Expression in Chickens

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SUMMARY. The epidermal growth factor receptor (EGFR), a growth-factor-receptor tyrosine kinase, is up-regulated in numerous tumors, which provides a good target for cancer therapy. Although it has been documented that oncoviruses are responsible for the activation of *EGFR* in tumors, the impact of Marek's disease virus (MDV) infection on *EGFR* has not yet been studied. We performed quantitative reverse transcriptase (RT)-PCR to check *EGFR* expression and found that it was significantly down-regulated after MDV infection. To explore the mechanism of *EGFR* repression, we examined the level of methylation of the *EGFR* promoter. The methylation level was significantly increased at 21 days postinfection, indicating a potential role of promoter methylation in *EGFR* repression.

RESUMEN. La metilación del ADN disminuye la expresión del receptor del factor de crecimiento epidérmico (EGFR) en los pollos.

El receptor del factor de crecimiento epidérmico (con las siglas en inglés EGFR), que es un receptor de un factor de crecimiento del tipo quinasa de tirosina, está regulado para su aumento en numerosos tumores, lo que lo convierte en un buen objetivo para la terapia contra el cáncer. Aunque se ha documentado que los oncovirus son responsables de la activación de EGFR en tumores, el impacto de la infección por el virus de la enfermedad de Marek (MDV), sobre el EGFR aún no ha sido estudiado. Se llevó a cabo una técnica de transcriptasa inversa y PCR cuantitativo (RT-PCR) para comprobar la expresión del EGFR y se encontró que era regulado hacia su disminución de manera significativa después de la infección por el virus de Marek. Para explorar el mecanismo de represión del EGFR, se examinó el nivel de metilación del promotor del EGFR. El nivel de metilación aumentó significativamente a los 21 días después de la infección, lo que indica un posible papel de la metilación del promotor en la represión del EGFR.

Key words: *EGFR*, promoter, methylation, Marek's disease virus, chicken

Abbreviations: EBV = Epstein-Barr virus; EGFR = epidermal growth factor receptor; HCV = hepatitis C virus; JAK/STAT pathway = Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway; PI3K-Akt pathway = phosphatidylinositol 3'-kinase (PI3K)-Akt pathway; Ras/MAPK pathway = Ras/mitogen-activated protein kinase (MAPK) pathway; RT = reverse transcriptase; TSS = transcription start site

The epidermal growth factor receptor (EGFR) (18) is a member of the HER/Erb-B transmembrane receptor tyrosine kinase family and functions in activation of multiple signaling pathways, including Ras/mitogen-activated protein kinase (MAPK), Src kinases, Janus kinase (JAK)-signal transducer and activator of transcription (STAT), and phosphatidylinositol 3'-kinase (PI3K)-Akt (4). The activation of EGFR by specific ligands, such as growth factor, controls multiple key cellular events, including proliferation, differentiation, locomotion, cell-cell interaction, survival, and apoptosis (17,26). The EGFR protein is distributed in normal tissues of mesenchymal, epithelial, and neuronal origin (25) but is up-regulated in some solid tumors (27), which indicates that the expression of *EGFR* needs to be maintained in some sort of balance to avoid its oncogenic threat. In normal tissues the abundance of *EGFR* mRNA is the main regulator of its expression (12,24). DNA methylation is another regulator of *EGFR* that could change the expression of *EGFR* in cancer (16). However, some oncoviruses, such as hepatitis B viruses and Epstein-Barr virus (EBV), encode regulators of *EGFR*, disturbing the balance of EGFR signaling (15).

Marek's disease virus (MDV) is an oncovirus, which induces lymphomas in susceptible chickens, also known as Marek's disease (3). Although the regulation of *EGFR* expression in human cancers was studied widely, little is known about the regulation of *EGFR*

by MDV in chickens. The *EGFR* gene in chicken encodes a glycoprotein that is about 170 kDa (7). EGFR is involved in chickens in the proliferation (8,23), development (1), and differentiation (19) of various organs, which is similar to its functions in humans.

In this study, based on the conserved function and sequence of *EGFR* in chicken, we hypothesized that the expression of *EGFR* is influenced by MDV infection. To test this hypothesis, we examined the expression of *EGFR* in chickens infected with MDV and found that the *EGFR* expression was significantly down-regulated by MDV infection. DNA methylation level of a CpG island overlapping with the *EGFR* promoter region indicated that DNA methylation altered the *EGFR* expression. This finding was further confirmed with an *in vitro* assay.

MATERIALS AND METHODS

Animals, virus infection, and sampling. Specific-pathogen-free white leghorn chickens were housed, challenged, and sampled in a Biosafety Level (BSL)-2 facility at the Avian Disease and Oncology Laboratory (ADOL, USDA, East Lansing, MI). A very virulent plus (vv+) strain of MDV (648A passage 40) was used to infect four chickens intra-abdominally with 500 plaque-forming units at 5 days of age. Another four chickens uninfected were used as age-matched control. Spleen samples were collected at 21 days postinfection (dpi), immediately placed in RNAlater (Qiagen, Frederick, MD), and stored at -80°C until used. All experimental chickens were managed and

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Table 1. Primer sequences for the experiments.

Primer ID	Sequence (5' to 3')	Product length (bp)	Purpose
EGFR-Pyro-F2	GGAGTAGTTGGGGTTAGTYGTG	134	Bisulfite pyrosequencing 2
EGFR-Pyro-R2	GGGACACCGCTGATCGTTATCCTCCCRTTCCCTTACCC		
EGFR-Pyro-S2	CRTGGGATTATTTGTTAG		
EGFR-Pyro-F3	GGATAGGGGGTTGCGTATATTAT	208	Bisulfite pyrosequencing 3
EGFR-Pyro-R3	GGGACACCGCTGATCGTTAACAAATCCGCCCGCAACT		
EGFR-Pyro-S3	TTTGCCTTCGTTTA		
EGFR-Pro-F1	GAAGATCTAGCGTCCCTGCTTGATAA	407	Amplify promoter region 1
EGFR-Pro-R1	CTAGCTAGCTTATTTGTTGGGGGTGGAA		
EGFR-Pro-F2	GAAGATCTTCACCCCCACAAAAATAA	824	Amplify promoter region 2
EGFR-Pro-R2	CTAGCTAGCCGTTCTCTCCTGTCCTG		
EGFR-Pro-F3	GAAGATCTCAGGACAGAGGAGAGGAACG	459	Amplify promoter region 3
EGFR-Pro-R3	CTAGCTAGCACCACTCAGAGCAGGGAGAA		

euthanatized following the ADOL Guidelines for Animal Care and Use (revised April 2005) and the Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research (ILAR) in 1996 (http://www.nap.edu/openbook.php?record_id=5140).

DNA extraction and promoter methylation analysis. DNA was extracted from 20 to 30 mg of spleen using DNeasy Blood & Tissue Kits (Qiagen). Bisulfite treatment of 500 ng DNA was performed using

EZ DNA Methylation-Gold Kit™ (ZYMO Research, Irvine, CA). Pyrosequencing was used to examine the DNA methylation level with the Pyro Q-CpG system (Biotage, Uppsala, Sweden) as previously described (2,28). The BigDye Terminator v3.1 Cycle Sequencing Kit (ABI, Carlsbad, CA) was used for ABI 3730 sequencing according to the manufacturer's instructions. The primers for pyrosequencing and ABI 3730 sequencing are shown in Table 1.

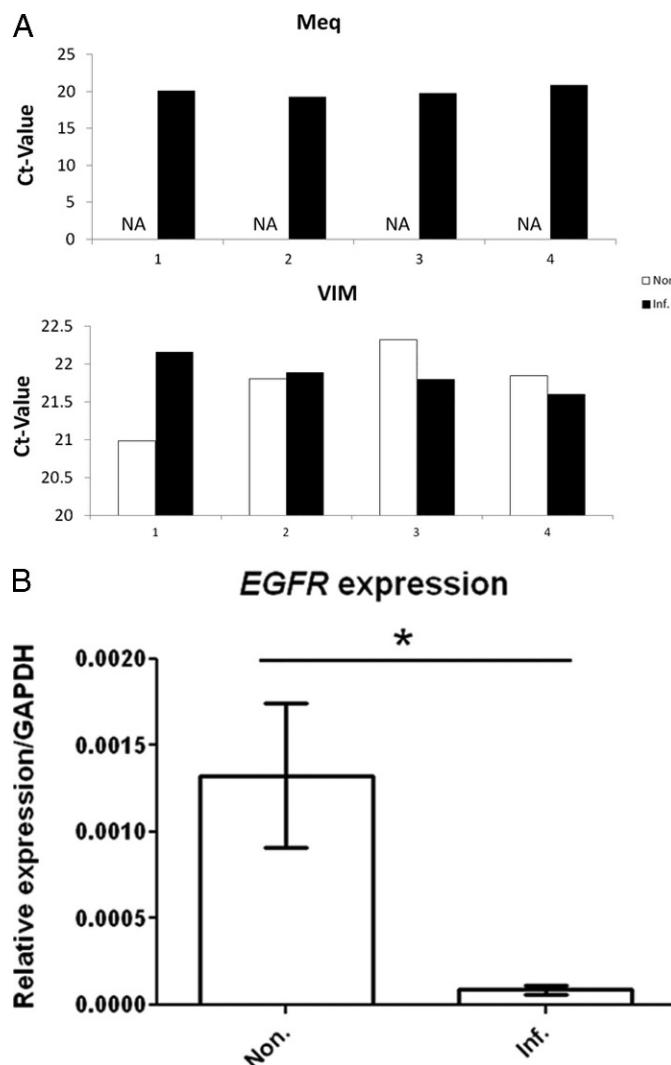


Fig. 1. Validation of MDV infection and *EGFR* expression detected by quantitative PCR. (A) The Ct value for Q-PCR of virus gene *Meq*, and a single-copy gene *Vim* in chicken. The x axis is the number of individual bird. Inf.: infected; Non.: noninfected. (B) *EGFR* expression before and after MDV infection as detected by quantitative RT-PCR. The samples were collected at 21 dpi. Data are shown as mean ± SEM. **P* < 0.05. *n* = 4.

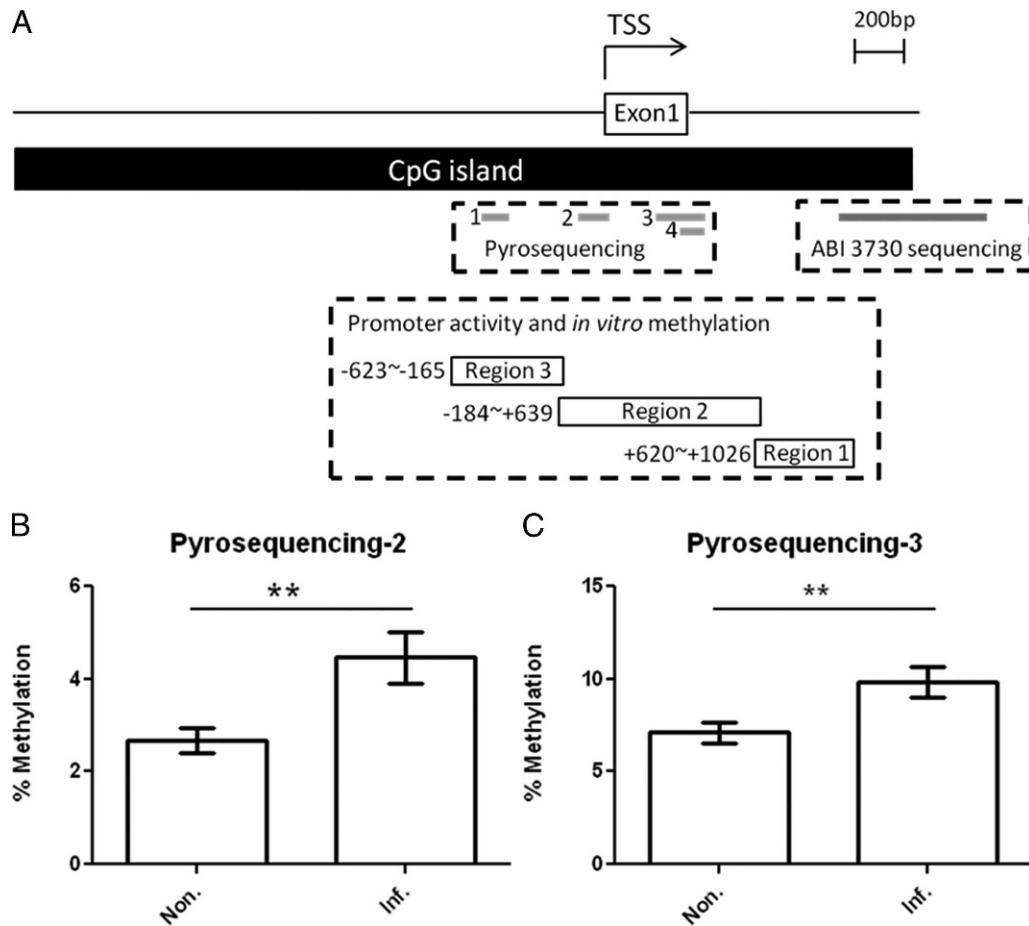


Fig. 2. DNA methylation-level analysis of *EGFR* promoter during MDV infection. (A) Schematic diagram of the entire CpG island around the TSS of the *EGFR* gene. The regions used for bisulfite sequencing and ABI 3730 sequencing are shown below the CpG island. The regions used for promoter activity analysis using luciferase reporter were also shown below. (B, C) DNA methylation levels were significantly up-regulated by MDV infection. Data are shown as mean \pm SEM. ** P < 0.01.

Viral load. The MDV gene *Meq* was used for quantification of viral genomic DNA in spleen. Quantitative PCR was performed by using 10 ng of genomic DNA on the iCycler iQ PCR system (Bio-Rad, Hercules, CA) and QuantiTect SYBR Green PCR Kit (Qiagen). The single-copy gene *Vim* (vimentin) (29) is used as internal control with the following primers: forward: 5'-CAGCCACAGACTGGTAGTC-3'; reverse: 5'-GAATAGGGAAACAGGAAAT-3'.

RNA extraction and quantitative real-time reverse transcriptase (RT)-PCR. RNA was extracted from 30 to 50 mg of spleen using RNeasy Mini Kit (Qiagen). Reverse transcription was carried out in 20 μ l with 1 μ g of total RNA with the QuantiTect Rev. Transcription Kit (Qiagen). The primers for the *EGFR* quantitative real-time (q)RT-PCR are as follows: forward: ATCGCGCTGGTGTCTCGG; reverse: GGGTGCCTCCGACGATA. qRT-PCR was performed on the iCycler iQ PCR system (Bio-Rad) in a final volume of 20 μ l using QuantiTect SYBR Green PCR Kit (Qiagen) with the following procedures: denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 30 s, then extended at 72 °C for 10 min. Each reaction was performed in triplicate. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward: GAGGGTAGTGAAGGCTGCTG; reverse: ACCAGGAAACAGCTGACG) was used to normalize the loading amount of cDNA.

In vitro methylation reactions, transfection assays, and luciferase assay. The promoter fragments of *EGFR* were obtained by PCR amplification by using three pairs of primers (Table 1). Then the *EGFR* promoter fragments were methylated by *Sss*I methylase (NEB, Ipswich, MA) following the manufacturer's instructions. Both the methylated and unmethylated *EGFR* promoter fragments were cloned into a

promoterless luciferase pGL-4.10 vector (Promega, Madison, WI). We used HeLa and DF1 cells to examine *in vitro* methylation reactions. Both cell lines were maintained in DMEM with 10% fetal bovine serum at 37 °C and 5% CO₂. One day before transfection, 1 \times 10⁵ HeLa and DF1 cells were seeded in 24-well plates and were about 70% confluent at 24 hr. Then the HeLa and DF1 cells were transiently transfected with 400 ng of unmethylated or methylated *EGFR* promoter pGL4.10-vector and 20 ng of a pRL-SV40 vector (as an internal control of transfection efficiency) using LipofectamineTM 2000 Transfection Reagent (Life Technology, Grand Island, NY). After 24 and 48 hr, both the firefly and renilla luciferase activity were measured by Dual-Luciferase[®] Reporter Assay System according to the manufacturer's protocol (Promega).

Transcription binding factor prediction. The transcription binding factor sites of *EGFR* promoter were predicted by PROMO 3.0, which recognizes sequences of the transcription binding factors as defined in the TRANSFAC database (13). The chicken transcription binding factors with a recognition sequence containing CpG were selected for the predictions.

RESULTS AND DISCUSSION

***EGFR* expression is down-regulated by MDV infection.** To check if the *EGFR* expression was altered by MDV, we did a qRT-PCR experiment using MDV-infected chicken spleen samples. To confirm that the chickens were infected with MDV, we first performed qPCR to determine the presence of the *Meq* gene, the

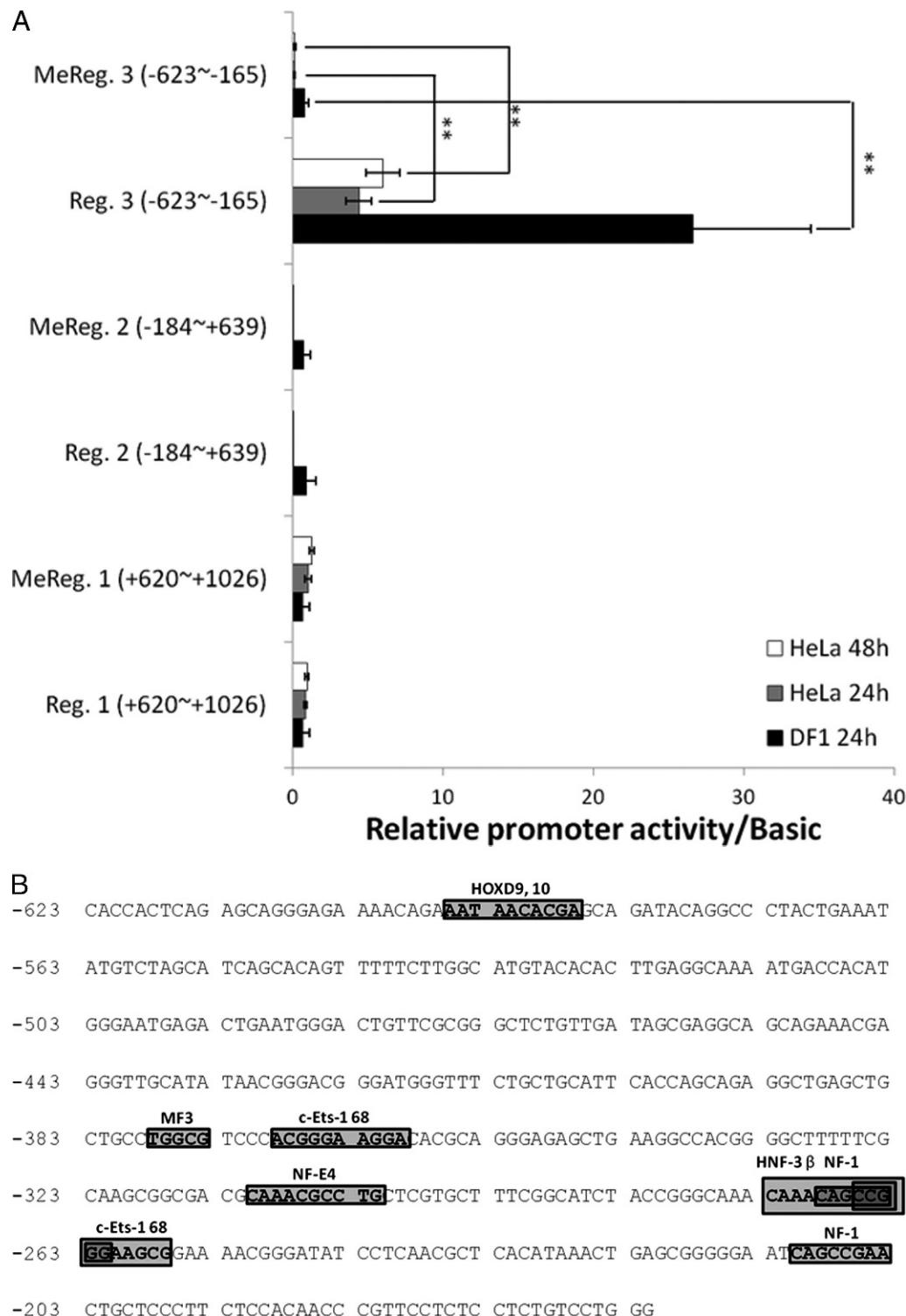


Fig. 3. DNA methylation represses the activity of *EGFR* promoter. (A) Promoter activity of the methylated and unmethylated control DNA fragments as indicated by the luciferase reporter. MeReg.1, MeReg.2, MeReg.3: methylated region 1, 2, and 3 DNA fragments around TSS of *EGFR* gene; Reg.1, Reg.2, Reg.3: unmethylated region 1, 2, and 3 DNA fragments. The numbers in the brackets indicate the relative position to TSS. Data are shown as mean \pm SEM. ** $P < 0.01$. $n = 4$. 1 (B) Transcription binding factor prediction by PROMO 3.0. The bold characters with dark shading are the predicted binding sequence for each transcription binding factor. The names of the predicted factors are marked on their recognition sequence.

MDV-associated oncogene. As shown in Fig. 1A, Meq DNA was not detected in the control samples, whereas similar copy numbers of the Meq gene were present in the infected samples as in the internal control *VIM* DNA. mRNA expression of *EGFR* was significantly

($P < 0.05$) down-regulated in the infected chickens compared to the controls. (Fig. 1B). Altered expression level of *EGFR* is a very common phenomenon observed in cancer cases and virus-infected samples (15). Thus, many cancer therapies were designed by

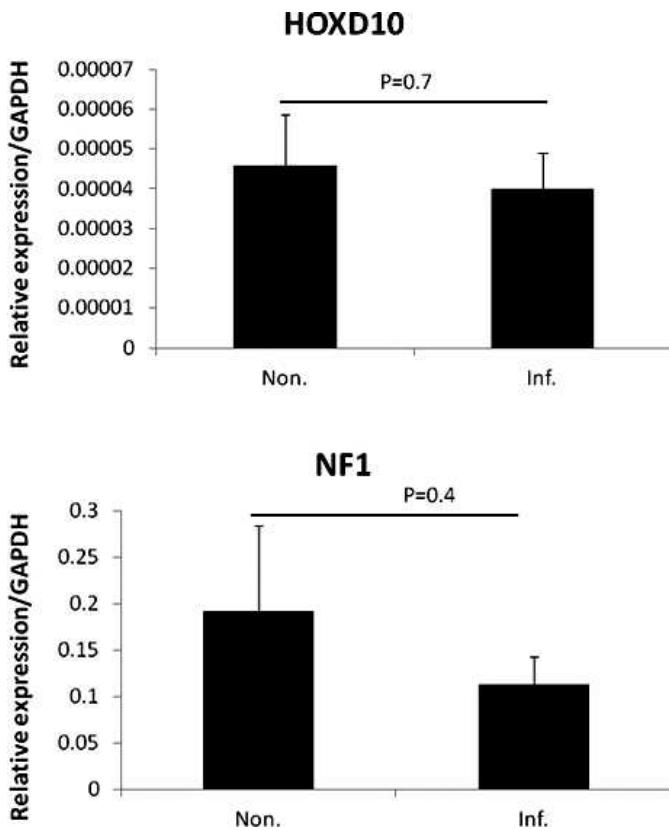


Fig. 4. The expression of two transcription factors HOXD10 and NF1 in spleen samples from MDV-infected (Inf) and noninfected (Non) chickens. Data are shown as mean \pm SEM. $n = 4$.

targeting the EGFR gene (20). Exploring the mechanism underlying the regulation of *EGFR* expression can provide additional information on the etiology of cancer. Oncovirus infection is one factor that can influence the *EGFR* balance. EBV latent membrane protein 1, an oncoprotein of EBV (5), activates *EGFR* expression through protein kinase C delta (6,14). Activation of EGFR is required for hepatitis C virus (HCV) infection, as EGFR is the host cofactor for HCV entry (11). However, our data from this study showed that the *EGFR* expression in chickens was down-regulated by MDV infection. Overexpression of *EGFR* in tumors can activate a large number of signaling pathways, such as Ras/MAPK, Src kinases, JAKs/STATs, and PI3K-Akt, which can induce the proliferation, survival, and invasion of cancer cells (4). It remains to be determined if the EGFR pathway is involved in the MDV-induced tumorigenesis by an alternative mechanism, which may lead to the down-regulation of *EGFR* expression upon MDV infection.

Increased EGFR promoter methylation in MDV-infected samples. It is well known that DNA methylation is a transcriptional repressor mechanism (18). Expression of *EGFR* was repressed by DNA methylation in some human cancers (16). Therefore we wanted to determine if DNA methylation was involved in the regulation of *EGFR* expression during MDV infection. Examining the chicken genome, we found a large CpG island that overlaps with exon 1 spanning about 3.6 kb around the transcription start site (TSS) of the *EGFR* gene (Fig. 2A). We used both bisulfite pyrosequencing and ABI 3730 sequencing to study the DNA methylation level of the partial CpG island around the TSS region (Fig. 2A). Two of the regions that we examined showed significantly ($P < 0.01$) higher methylation levels in the infected samples

compared to the noninfected (Fig. 2B, C). Combined with the expression result, we could see a repression effect on gene expression by the increased promoter methylation. Combined with our previous finding that MDV-induced DNA methylation changes subsequently influence the expression of corresponding genes (9,10), we found that MDV can induce a variety of methylation changes in chicken and consequently alters gene expression. The increased promoter methylation level of *EGFR* may result from the increased expression of DNA (cytosine-5)-methyltransferase 1 (*DNMT1*) (9). However, when we compared the DNA methylation change rate with that of expression change, we found the former one is much less than the latter one. Although the relationship between DNA methylation change and gene expression is unclear, we hypothesize that DNA methylation may not be the only factor causing down-regulation of *EGFR* expression by MDV infection.

Inactivation of *EGFR* promoter activity by DNA hypermethylation *in vitro*. To ascertain whether increased methylation levels of the promoter have a negative relationship with *EGFR* gene expression, an *in vitro* methylation experiment was conducted using luciferase as a reporter. Because there is no information regarding the active chicken *EGFR* promoter, we first chose three regions around the *EGFR* TSS to assess the promoter activity and methylated the three regions *in vitro* to test if the promoter activity was influenced (Figs. 2A and 3A). Only region 3 (from -623 to -165 upstream of the TSS) showed promoter activity in HeLa and DF1 cells with a higher promoter activity in DF1 cells than in HeLa cells (Fig. 3A), which further indicates the chicken origin of these sequences. After methylation, the promoter activity of region 3 was significantly ($P < 0.01$) repressed. However, no significant changes were detected in the other two regions ($P > 0.05$). The results were similar for the 24 and 48 hr treatments. This result indicates that DNA hypermethylation can inactivate the promoter activity of *EGFR*. Subsequently, we analyzed which transcription factor binding sites in region 3 may be affected by the DNA methylation. Several transcription factors binding sites with the recognition sequence containing CpG were found in region 3, including MF3, HOXD9, HOXD10, NF-1, c-Ets-1 68, HNF-3 β , and NF-E4, (Fig. 3B). If the expression of EGFR was down-regulated only by the promoter methylation, the expression of transcription factors may stay the same after MDV infection. qPCR analysis of HOXD10 and NF1 showed that the expression of these transcription factors was not altered by MDV infection (Fig. 4). It is highly likely that two factors regulate EGFR expression by DNA methylation: first, the methylation on TFBS prevents the binding of the transcription factors (22); second, some proteins, such as methyl CpG binding protein 2 (MeCP2), bind to the methylated DNA region occupying the TFBS (21). However, due to the lack of extensive studies on the regulation of *EGFR* expression, little is known about the function of these transcription factors in EGFR signaling pathway. Therefore, further work is needed to determine how *EGFR* expression is regulated by DNA methylation.

Our data from this study showed that MDV infection altered the balance of *EGFR* expression in chickens by significant down-regulation. Further analysis of the CpG island in the promoter region of *EGFR* showed an increase in DNA methylation, indicating a repression effect on *EGFR* expression, which was confirmed by an *in vitro* methylation assay. In conclusion, MDV infection accelerated DNA methylation, which down-regulated *EGFR* expression.

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